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MRI Project No. 2688-B

Contract No. DA-18-108-AMC-120(A)

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BIMONTHLY PROGRESS REPORT NO. 5

Covering the Period

1 December 1963 - 31 January 1964

RESEARCH, DEVELOPMENT AND FABRICATION OF AN EXPENDABLE BIOELECTROCHEMICAL DETECTOR SYSTEM

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Prepared by

Elven K. Bauman

19 February 1964

PREFACE

This report describes the work performed during the bimonthly period ending 31 January 1964 on Contract No. DA-18-108-AMC-120(A).

The experiments were conducted by Mr. Elven K. Bauman, Project Leader, and Mrs. Rosalie Hudson. Mr. Clayton C. Craghead, Assistant Electrical Engineer, has provided information regarding solid-state read-out devices. Dr. Louis H. Goodson, Principal Biochemist, assisted in the planning of the experiments and in the evaluation of the results obtained.

Approved for:

MIDWEST RESEARCH INSTITUTE

W. B. House, Director
Biological Sciences Division

19 February 1964

TABLE OF CONTENTS

SUMMARY

This report describes the use of open cell urethane foam pads for supporting the starch immobilized enzyme. Eight per cent starch gel is prepared and enzyme added as the gel cools. Subsequently, foam pads are saturated with the gel, the excess gel squeezed out, and the pads freeze-dried. Experiments show that pads prepared in this manner and exposed to a stream of 1 ml. of substrate and 1-1/2 liters of air per minute will continue to hydrolyze substrate in excess of 2 hr.

This report also describes the operation of an experimental detector for a period of 2 hr. and the response of the detector to an enzyme inhibitor. This test employed perforated grid electrodes fabricated from platinum sheet. These electrodes were in contact with the surfaces of the foam pad.

The feasibility of bioelectrochemical monitoring of enzyme activity as a method for detecting nerve agents is demonstrated.

I. INTRODUCTION

The objectives of this research program are to develop and to build a bioelectrochemical detector for various nerve agents. The method of choice for this device is the constant current polarographic technique described by Kramer, Cannon, and Guilbault.[1]

Our efforts during the fifth bimonthly period have been devoted to studies related to (1) supporting the immobilized-enzyme by open-cell urethane foam, and (2) the use of grid electrodes in an experimental detector.

II. ENZYME SUPPORT

The use of starch-gel-coated polyurethane shreds for supporting the immobilized enzyme was mentioned in Bimonthly Progress Report No. 4 of this project. Further experience with urethane shreds led to the use of open cell urethane foam. Initially, 1/4 in. foam sheet was cut into rough circles approximately 1 in. in diameter. These circles were saturated with starch gels of various concentrations, the excess gel squeezed out, and the circles freeze-dried. These first foam pads weighed about 90 mg., and after coating and freeze-drying, were found to retain 10 - 20 mg. of starch, depending upon the per cent composition of the starch gel.

The porosity of the pads was investigated by placing the pads in a Millipore microanalytical filter holder[2] with grid type platinum electrodes above and below the pad (see pages 5 and 6 of this report). The filter holder was held together with the clamp provided with the filter. At a differential pressure of 2 in. Hg, we observed air flow rates on the order of 1 liter/min simultaneous with a flow of 1 ml. of liquid per minute even with the pads somewhat crushed by the clamping. For these experiments we used a Brailsford[3] Model TD-1 Blower. Table I presents the relationship between starch gel composition, retention of starch, and pad porosity. Based on the liquid and air flow data available at present, we have tentatively selected 8 per cent starch gel for coating the foam pads.

1/ Kramer, D. H., P. L. Cannon, Jr., and G. C. Guilbault, Anal. Chem., 34, 842-845 (1962).

2/ Millipore Filter Corp., Bedford, Massachusetts.

3/ Brailsford and Company, Inc., Milton Point, Rye, New York.

TABLE I

THE RELATIONSHIP BETWEEN STARCH GEL COMPOSITION, STARCH RETENTION AND PAD POROSITY

Number	% Starch	Wt. Gel (mg.)	Wt. Enzyme (mg.)	Air Flow*
1	4	4	0.09	3.6 liters/min at 3 in. Hg
2	4	10	0.24	3.3 liters/min at 3 in. Hg
3	4	12	0.28	3.3 liters/min at 3 in. Hg
4	8	11	1.1	1.8 liters/min at 2 in. Hg
5	8	11	1.1	-
6	8	11	1.1	1.7 liters/min at 2 in. Hg
7	8	13	1.3	-

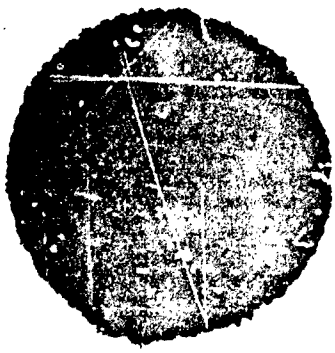
* Air flow simultaneous with 1 ml. of substrate per minute.

In addition to the effect on starch retention and pad porosity, the concentration of the starch gel applied to the pad undoubtedly affects the resistance of the immobilized enzyme to leaching during detector operation and the proportion of the immobile enzyme that is exposed and available to hydrolyze the substrate stream. Investigation of these factors is beyond the scope of the present contract.

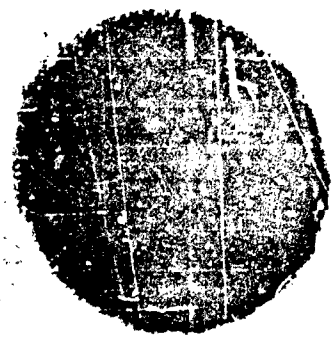
In order to gain some idea of the uniformity of the starch on the pads and the resistance of the starch to erosion by the liquid stream, we stained several pads with I_2·KI solution.[4] Photographs of three of these pads are shown in Fig. 1. The pads A and B in Fig. 1 were prepared as previously described. Pad C was not treated with starch gel and is included for visual comparison. Pad A is unused and illustrates the uniform distribution of the starch gel on the urethane foam. The color after the application of the I_2·KI solution was the typical purple-black of the starch-I^- complex. Pad B was used in an experimental detector for approximately 1 hr. during which time it was exposed to a flow of 1.5 liters of air and 1 ml. of substrate solution per minute. Thereupon, the pad was stained with I_2·KI solution and again the typical purple-black color is observed. This test indicates that much of the starch is held in the pad during detector operation. Subsequent work described later in this report demonstrates that a significant portion of enzyme is also retained in the pad.

[4] 0.1 M I_2 in 0.1 N KI.

Pad A - Unused, starch-coated pad.



Pad B - Starch-coated pad after 1 hr. operation in detector at 1 ml. solution and 1.5 liters of air flow per minute.



Pad C - Control pad without starch gel.

Fig. 1 - Photographs of $I_2 \cdot KI$ Stained Urethane Foam Pads Before and After Use (2-1/4 X) See Text

Operation of the experimental detector with pads 1, 2, and 3 (Table I) demonstrated that the amount of enzyme in each pad was insufficient to hydrolyze the 5×10^{-4} M·BuSChI substrate stream. Pads 4, 5, 6, and 7 were prepared with a gel designed to leave 1 mg. of enzyme on each pad. Operation with these latter pads was successful from the standpoint of hydrolyzing a major portion of the substrate stream. However, the time of response to simulated enzyme inhibition was excessive (3 - 6 min.). It appeared as though clamping of the enzyme-containing pads and electrodes in the filter holder and the crushing action attendant thereto caused channeling and stagnation of liquid held in the pad. To circumvent these problems, we cut foam pads 3/4 in. diameter to fit into 1 in. x 3/4 in. x 1/8 in. O-rings. The 1/8 in. thickness of the O-ring prevents complete crushing of the pad and at the same time permits compression of the 1/4 in. foam to 1/8 in. The latter insures good contact between the platinum grids and the pad surfaces. In addition to decreasing response time, we found that the air flow rate was increased about 50 per cent by using the O-rings. Figure 2 illustrates the cross-section of the filter holder with the O-ring, filter pad, and electrodes in place.

Another approach to the problems of reducing stagnation and response time is the use of platinum electrodes made from gauze or perforated sheet to provide more uniform distribution of the substrate on and within the pad. We have ordered perforated platinum sheets with 576 0.032 in. holes/sq in and with 152 0.062 in. holes/sq in to investigate this type of electrode.

III. DETECTOR OPERATION

Operation of the experimental detector has been used to (1) compare the signal obtained from wire electrodes with that obtained from grid electrodes, and (2) evaluate starch-enzyme formulations on the basis of pad porosity, enzyme content, and enzyme retention. The apparatus was set up as shown in Fig. 3. The circuit shown can be connected to either the wire or the grid electrodes. We used a Kay Lab Model 203 microvolt ammeter to measure both voltage and current since this particular instrument has provisions for separate voltage and current inputs and the meter can be switched from one mode to the other without moving test leads or changing the electrical characteristics of the circuit under investigation. Moreover, the Kay Lab Model 203 has an output circuit to drive a recorder and thus isolate the input impedance of the recorder from the detector circuit.

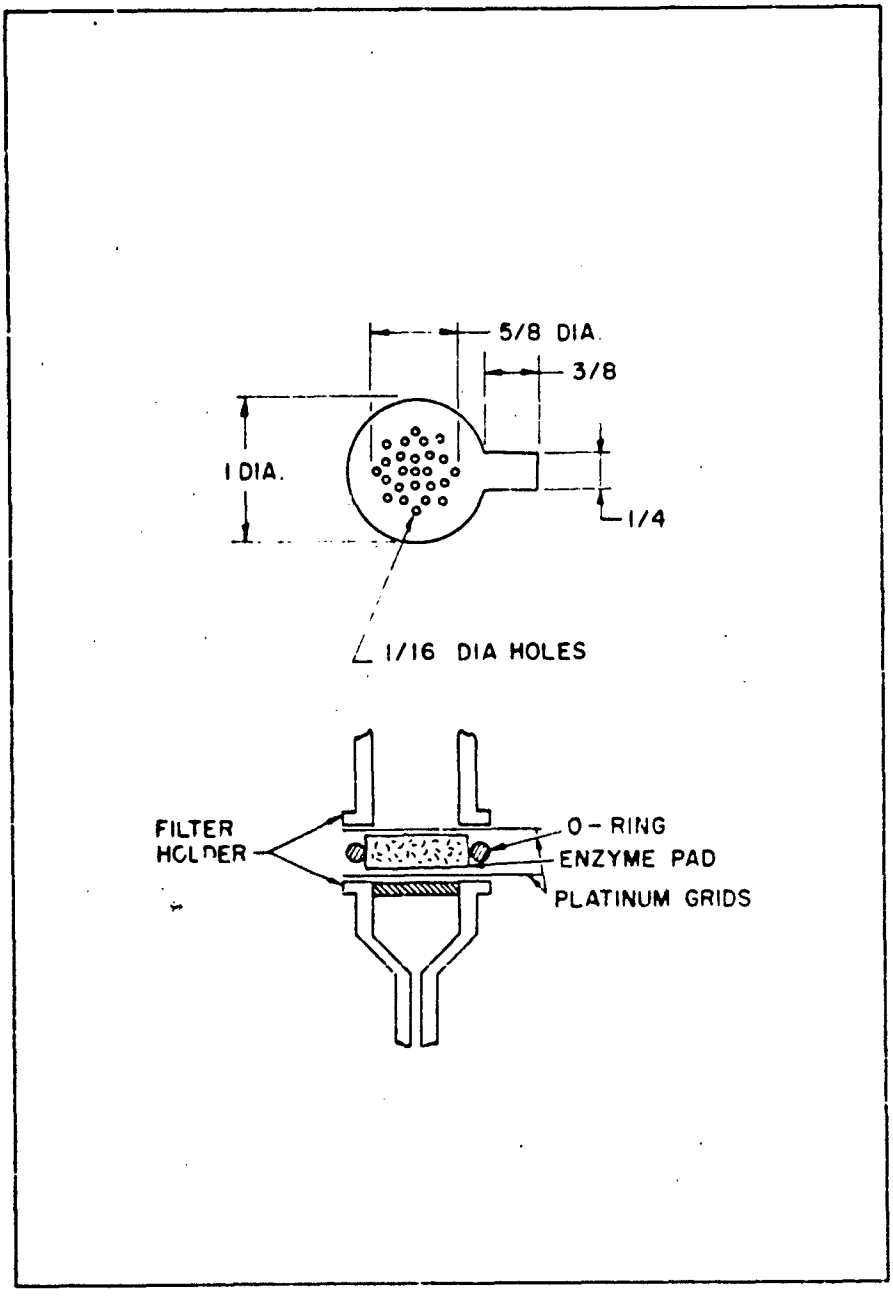


Fig. 2 - Details of Enzyme Pad, O-ring and Grid-Electrode Assembly

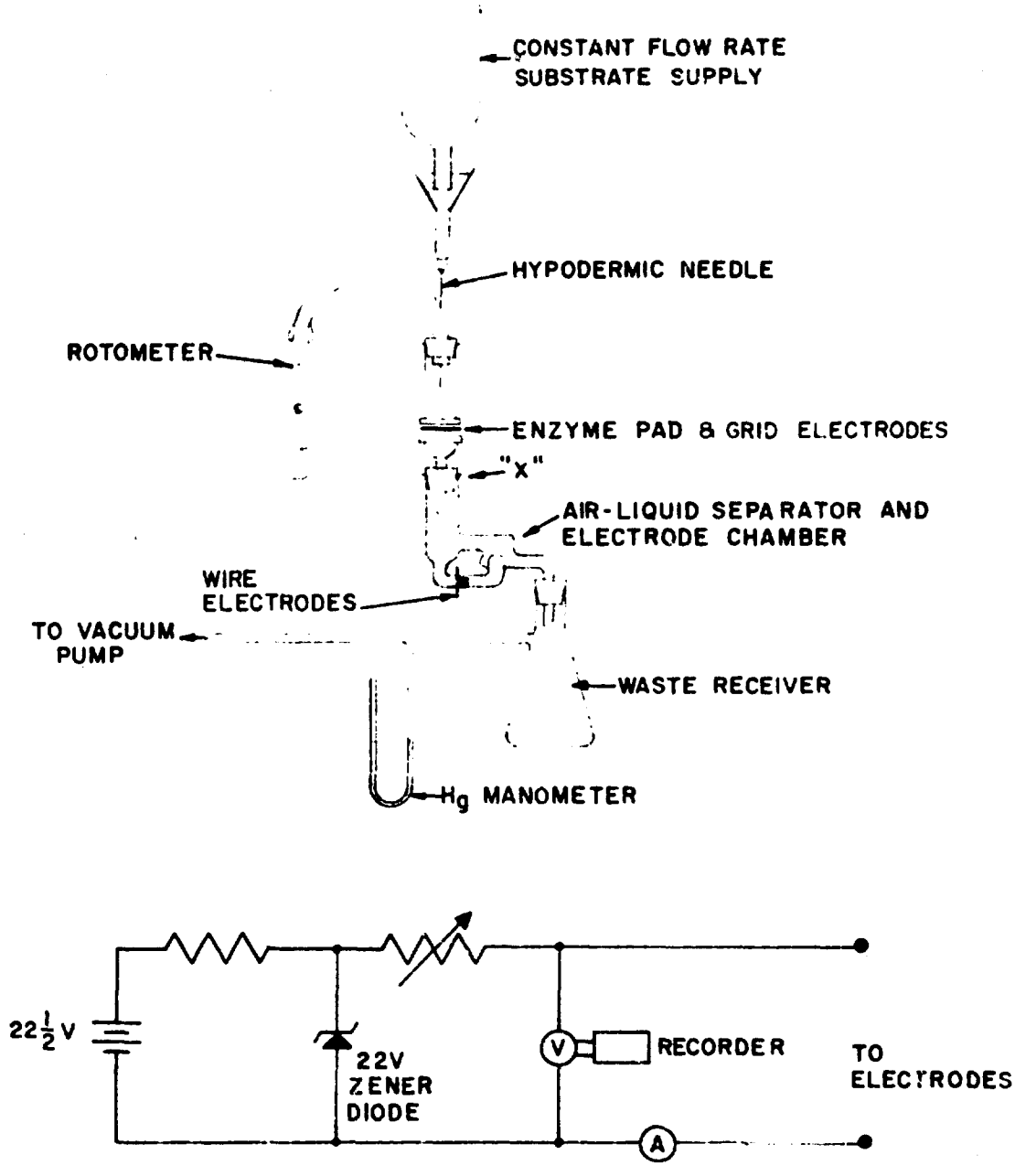


Fig. 3 - Experimental Detector Apparatus and Associated Circuitry

The following steps were used to establish detector operation:

1. Establish reference voltages. A foam pad without enzyme was placed in the filter holder with the grid electrodes and the holder was clamped together. The air stream and the substrate stream were started. While the electrodes were exposed to flowing substrate the cell voltage was recorded as "alarm-voltage." The liquid stream was then changed to prehydrolyzed substrate (thiol) and the cell voltage recorded as "safe-voltage." Finally, the substrate stream was restored, whereupon the meter returned to "alarm-voltage."

2. Insertion of enzyme pad. The substrate flow was interrupted and the electrical circuits disconnected during the replacement of the enzyme-free pad with an enzyme-containing pad.

3. Initiation of operation. With an enzyme-containing pad in place and detector operation started, the cell voltage was recorded as "operating voltage." Occasionally it was desirable to switch the liquid stream to prehydrolyzed substrate (thiol) temporarily in order to recheck safe voltage.

4. Check of detector operation. To simulate the sampling of an inhibitor aerosol, a solution of an enzyme inhibitor was incorporated into the liquid stream. The cell voltage after inhibition was recorded as "inhibited voltage."

Polarograms of 5×10^{-4} M substrate and hydrolysis products made previously using grid electrodes revealed that at 2 μa. we could expect a voltage of 475 mv. during substrate flow and 225 mv. during the flow of hydrolysis products. These voltages were not attained in the initial tests of the detector. As indicated earlier in this report we believe this situation was due to incomplete hydrolysis of the substrate by the pad caused by the crushing of the foam pad by the filter holder and clamp. In subsequent operations of the detector during which the pad was protected by an O-ring, the results were more consistent with the polarographic data. The operating conditions and observations during this test are presented in Table II.

We believe that the test operation of the detector described in Table II fully substantiates the feasibility of the bioelectrochemical approach to the enzymatic detection of nerve agents. This test demonstrates: (1) Potentiometric surveillance of the hydrolysis of a substrate stream by an immobilized enzyme, (2) rapid potentiometric response to inhibition of the enzyme, and (3) the operation of the detector system in excess of 1 hr. with the possibility of unattended operation for several hours.

TABLE II

OPERATING CONDITIONS AND OBSERVATIONS DURING DETECTOR TESTS

I. Substrate: Butyrylthiocholine iodide
Conc. 5 x 10^{-4} M
Vehicle tris buffer 0.1 M pH 7.4
Flow rate 1 ml/min

II. Enzyme: Serum cholinesterase
Amount: 0.96 mg. total in pad (see discussion, Section II)
Support: 3/4 in. diameter x 1/4 in. urethane foam, in O-ring

III. Atmosphere: Room air
Flow rate: 1.7 liters/min
Differential pressure: 2 in. Hg
Pump: Brailsford TD-1 blower

IV. Circuit: Constant current polarography
Electrodes: Platinum grids
Current: 2 μa.

V. Inhibitor: Eserine (physostigmine)
Amount: ca. 100γ total
Vehicle: Water 1 ml.

VI. Observations:

A. Voltage across electrodes

1. Reference	(a) alarm	410 mv.
	(b) safe	225 mv.
2. Operation		275 mv.
3. After inhibitor		390 mv.

B. Duration of operation
1. Prior to inhibition - 1-3/4 hr.
2. After inhibition - 3/4 hr.

C. Response to inhibitor

Elapsed time (sec.)	% of voltage change
15	50
30	70
45	80
60	90
75	95
90	100

IV. DISCUSSION

At the close of the fifth bimonthly period we have demonstrated the feasibility of bioelectrochemical monitoring of enzyme activity as a method for detecting nerve agents.

During a project meeting at Edgewood Arsenal on 20 January 1964 between Mr. Bauman of the Institute, Dr. George Guilbault, cognizant CRDL Project Officer, and Dr. D. N. Kramer, Chief, CRDL Detection Research Branch, it was agreed that the nature of this research program was such that the step-wise phase attack on the problem was not practical since much of the Phase I work hinged upon the prior completion of portions of the Phase II and Phase III objectives. On this basis, it was further agreed that Phases I, II, and III had been undertaken simultaneously and they are considered to be complete as of 31 January 1964.

V. FUTURE WORK

The final bimonthly period of this contract will be devoted to the objectives of Phase IV, namely the fabrication of two experimental detectors for submission to CRDL at the close of the contract. We plan to build two units. One unit will be provided with an air-liquid separator and wire electrodes, the second unit will incorporate the grid electrodes. Each unit will be complete with a rotometer for measuring air input, the circuitry related to the electrodes, a blower and mercury manometer. The units will have suitable connections to provide 24 v. DC input as well as cell voltage and current output terminals.

Once the units have been completed and tested, they will be delivered to CRDL where Mr. Bauman will demonstrate their operation.

In addition to the experimental detector, we plan to supply a number of foam pads with gel and enzyme as our supply of enzyme permits.

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